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IMMUNE MODULATION BY COXIELLA BURNETII: CHARACTERIZATION OF A PHASE I IMMUNOSUPPRESSIVE COMPLEX DIFFERENTIALLY EXPRESSED AMONG STRAINS

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ABSTRACT

Coxiella burnetii, the etiological agent of Q fever, possesses immunomodulatory activity which positively and negatively regulates host immune responses. We wish to determine the Coxiella strain differences and the chemical nature of cellular components suppressing lymphocyte responsiveness. The bacterial components responsible for the immunomodulatory activity are associated with phase I cells. In its natural state, the phase I cell-associated, immunosuppressive complex (ISC) was resistant to chemical and enzymatic treatment. The ISC was inactivated and rendered accessible by chloroform-methanol (CM) (4:1) extraction of phase I cells which produced a CM residue (CMRI) and CM extract (CME). The suppressive components in either CMRI or CME did not induce ISC activity in the host when injected separately. Reconstitution of the CMRI with CME prior to injection produced the same pathological reactions characteristic of phase I cells. The CMRI suppressive component was sensitive to alkali, acid, periodate, lysozyme, and neuraminidase, but resistant to lipase and protease. An active component of CMRI was attached to the cell matrix by disulphide bonds. The amphipathic,

lipophilic, CME suppressive component was ubiquitously distributed in procaryotes and eukaryotes because ISC activity of CMRI was regained after association with reagent-grade lipids and different CMEs. The ISC was expressed by phase I strains with smooth lipopolysaccharide (LPS) but not by phase II strains with rough LPS. Phase I heart valve strains carrying significant amounts of rough LPS did not express all of the biological properties of the ISC. The LPS molecule induced immune enhancement without immunosuppression. Thus, expression of the ISC showed strain variation and may be under genetic control. The complete details of the chemical composition and active components of the ISC should prove useful for biological-response-modification studies.

INTRODUCTION

The modulation of host immune responses after the injection of viable or inactivated phase I Coxiella burnetii, the etiological agent of Q fever, is expressed as both enhancement and suppressive activities. Immune enhancement by adjuvant-active components results in increased antibody production (1) and non-specific stimulation of macrophage functions (2,3). Immune suppression was shown by components that induce mitogenic hyporesponsiveness and antigen-specific negative modulation of murine splenic and nodal lymphocytes (4,5,6). Inactivation or disruption of the components responsible for the immunosuppressive pathogenic activity (i.e. an immunosuppressive complex, ISC) was accomplished by extraction of phase I cells with chloroform-methanol (4:1) to produce a particulate chloroform-methanol residue (CMR) and a soluble CM extract (CME) (4). When individual fractions of the ISC were tested in mice they did not induce pathological reactions. The CMR fraction was efficacious as a vaccine and the CME was ineffective (6).

The biologically active components of the ISC were further investigated by testing the hypothesis that recombining the phase

I CMR (CMRI) with the phase I CME (CMEI) would restore the pathogenic activity of the CMRI to that of phase I whole cells (WC). Indeed, the biological activity of the ISC of phase I cells was regained by reconstitution of CMRI with CMEI in the CM solvent (6). Therefore, we assumed that the biologically active ISC was composed of at least three components; one of which resided in CMEI, one associated with CMRI, and one attaching the former two components to the cell matrix.

In the present study, we investigated the chemical, structural association of the components participating in the ISC. It has been found that the ISC contains carbohydrate residues which may be attached to the cell by disulphide bonds. Furthermore, the ISC is a structural feature of phase I Nine Mile, phase I Henzerling, phase I Nine Mile 514, and phase I Ohio strains. The properties of the ISC were expressed differently by three phase II strains. We also show, for the first time, that mice injected with phase I strains isolated from heart valves of individuals with chronic Q fever endocarditis did not show immunosuppression.

MATERIALS AND METHODS

Coxiella burnetii Antigens

The C. burnetii strains were the Nine Mile [phase I clone 7 (CB9MIC7), phase II clone 4 (CB9MIIC4), and phase I 514 (CB9MI514)], the cardiotrophic [KAV phase I (CBKAVI) and PAV phase I (CBPAVI)], the M44 phase II (CBM44II), the Henzerling phase I

(CBHENI), the Australian phase II (CBAUSTII), and the Ohio phase I (CBOI) (7). The microorganisms were propagated in the yolk sacs of embryonated chicken eggs, purified, and inactivated with formaldehyde (8).

Chloroform-Methanol Extraction

Chloroform-methanol extraction of lyophilized C. burnetii cells, Legionella pneumophilia (9), chick embryo yolk sacs, and C57BL/10 ScN spleen cells with chloroform-methanol (CM) (4:1) (4) produced particulate components [chloroform-methanol residue (CMR)] and extracted components [chloroform-methanol extract (CME)].

Reconstitution of ISC

Reconstitution of lyophilized CMR with CME (or other biochemicals) was performed by suspending 400 μg of CMR (w/w) in 100 μg of CME. This ratio (4:1) was used because previous studies showed that CME is 20% by weight of the whole cells (4). The suspension was sonicated in an ultrasonic cleaner (model 8845-30; Cole-Palmer) for 5 min. The CM was evaporated by incubation overnight at room temperature or dried under a light stream of nitrogen. The residue was resuspended by ultrasonic treatment in sterile phosphate-buffered saline (PBS) to a concentration of 200 μg/ml. Reconstitution of different fractions before or after treatment (see below) was performed as described above. Phase I C. burnetii CMR was also reconstituted with L. pneumophilia CME,

chick embryo yolk sac CME, C57BL/10 ScN mouse spleen CME, CB9MIIC4-CME, phosphatidyl serine, phosphatidyl choline, phosphatidic acid, glycerol phosphate, glycerol, cholesterol, cardiolipin, Escherichia coli 2-keto-3-deoxyoctonic acid (KDO), pentadecanoic (C-15) and heptadecanoic (C-17) fatty acids, phosphatidyl glycerol, phosphatidyl glycerol dioleoyl, phosphatidyl ethanolamine, and phosphatidyl ethanolamine dipalmitoyl. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Inactivation of ISC

In certain experiments, CB9MIC7-WC and CBOI-CMR were enzymatically treated with proteinase K, thermolysin, lysozyme, beta-galactosidase, lipase, glucose oxidase, neuraminidase, DNase, or RNase (Sigma) according to protocols outlined in Boehringer-Mannheim procedure manuals (10,11). The particulates were collected after centrifugation at 100,000 x g for 30 min, washed with water three times, and lyophilized.

The susceptibility of the ISC to inactivation by reducing agents was tested by incubating CB9MIC7-WC and CBOI-CMR in 100 mM dithiothreitol (DTT) in PBS at 25°C for 1 hr. To selected fractions, 1 M iodoacetamide (IA) was added to a final concentration of 0.05 M to alkylate-reduced sulfhydryls to prevent them from reassociating. Treated CMR was reconstituted with C. burnetii phase I CME prior to mouse injection. In certain experiments, CMR was reconstituted with CME prior to DTT and

iodoacetamide treatment. In a variation of these procedures, the supernatant and residue after DTT extraction of CBOI-CMR were reconstituted with C. burnetii CME prior to mouse injection.

Fractionation of CME Components

Phase I CME of C. burnetii was passed through single-phase extraction columns (Baker Chemical Co., Phillipsburg, NJ) (12). One hundred micrograms of CME was applied to the columns. After 2-min adsorption, the columns were washed with CM, and the eluate was reconstituted with CBOI-CMR prior to injection into mice.

Mice

Endotoxin non-responder (13,14) mice (C57BL/10 ScN, Harlan Sprague-Dawley, Inc., Indianapolis, IN) were injected i.p. with 0.5 ml of saline containing 100 µg of various prepared fractions described above.

Immunological Assays

Nucleated cells obtained from the spleens of mice 14 days after injection were used for lymphocyte proliferation assays (1).

RESULTS

Fractions Modulating the Immune Response

Phase I whole cells (WCI) and phase I CMR (CMRI) reconstituted with phase I CME (CMEI) induce splenomegaly and lymphocyte hyporesponsiveness to mitogens and antigens (4,5,6).

TABLE 1

Comparison of the Mitogenic Responses of Lymphocytes from C57BL/10 ScN Mice Injected with C. burnetii Cells and Fractions

Exp.	Mice injected with[a]	Spleen wt.[b]	Response as Incorporation of [^{3}H]TdR			
			ConA		PWM	
			SI[c]	% Supp.[d]	SI	% Supp.
1	Saline	102.1 ± 5.6	90		29	
	WCI	724.5 ± 41.7	3	96	2	94
	WCII	120.3 ± 17.0	82	8	30	-5
	CMRI + CMEI	352.4 ± 32.8	7	92	1	95
	CMRII + CMEI	93.8 ± 5.7	60	33	21	25
	CMRI + CMEII	393.3 ± 79.0	8	91	2	94
	CMRII + CMEII	110.2 ± 2.8	71	21	23	21
	LPSI + CMEI	97.2 ± 13.1	67	26	24	18
2	Saline	101.6 ± 6.9	68		28	
	CMRI	93.1 ± 4.2	80	-18	32	-14
	CMRII	94.5 ± 10.7	96	-41	34	-23
	CMEI	111.5 ± 20.3	90	-34	30	-6
	CMEII	98.2 ± 14.4	67	1	27	3
	LPSI	120.3 ± 6.6	111	-63	31	-13

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 100 µg of C. burnetii cells, 80 µg of CMR, 20 µg of CME, 80 µg of LPS, or a reconstituted mixture of CMR (80 µg) plus CME (20 µg).

Different fractions, including samples reconstituted with CME, were tested in C57BL/10 ScN mice for their ability to induce splenomegaly and modulate the response of splenic lymphocytes to antigens and mitogens in culture (Table 1). Injection of 100 µg of WCI and WCII resulted in significantly increased spleen weights by the former but not by the latter. The injection of phase I lipopolysaccharide (LPSI) induced splenomegaly, while CMRI, CMRII, CMEI, and CMEII caused no significant increases in spleen weight.

Reduction in response to ConA and PWM was greater than 90% after the injection of WCI, CMRI plus CMEI, and CMRI plus CMEII. There was slight mitogenic hyporesponsiveness after injection of

TABLE 2

Comparison of the Antigenic Responses of Lymphocytes from C57BL/10 ScN Mice Injected with C. burnetii Cells

Exp.	Mice injected with[a]	Response as Incorporation of [^{3}H]TdR							
		WCI		WCII		CMRI		CMRII	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.	SI	% Supp.
1	Saline	4.73		9.68		4.12		18.70	
	WCI	0.27	94	0.25	97	0.18	96	0.79	96
	WCII	6.26	-32	13.03	-35	4.84	-17	19.45	-4
	CMRI + CMEI	0.60	87	0.72	93	0.45	89	1.00	95
	CMRII + CMEI	5.49	-16	10.05	-4	4.56	-11	11.87	37
	CMRI + CMEII	0.74	84	0.92	90	0.63	85	1.04	94
	CMRII + CMEII	4.59	3	8.28	14	3.42	17	12.17	35
	LPSI + CMEI	5.36	-13	9.75	-1	4.26	-3	12.04	36
2	Saline	4.06		8.34		3.22		12.09	
	CMRI	4.94	-22	11.26	-35	4.66	-45	21.68	-79
	CMRII	5.75	-42	11.85	-42	4.99	-55	27.48	-127
	CMEI	4.14	-2	12.08	-45	3.74	-16	18.61	-54
	CMEII	4.53	-12	10.63	-27	3.86	-20	10.07	17
	LPSI	4.37	-8	15.10	-81	3.91	-21	24.64	-104

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 100 µg of C. burnetii cells, 80 µg of CMR, 20 µg of CME, 80 µg of LPS, or a reconstituted mixture of CMR (80 µg) plus CME (20 µg).

CMRII plus CMEI, CMRII plus CMEII, and LPSI plus CMEI. When non-reconstituted component fractions were tested, we saw an increase in the mitogenic response with all but the CMEII extract.

Reduction in the ability of in vivo primed splenic lymphocytes to respond to recall antigens in vitro was greatest after the injection of WCI, CMRI plus CMEI, and CMRI plus CMEII (Table 2, Exp. 1). The injection of CMRII plus CMEI, CMRII plus CMEII, and LPSI plus CMEI induced significant antigen-specific suppression with only the CMRII recall antigen. However, we observed either no change or slightly decreased responses in vitro in the presence of the other recall antigens. All of the

Table 3

Effect of Enzymatic and Chemical Treatment on the Ability of CMRI to Reconstitute Immunosuppression with CMEI

Exp.	CMRI treatment[a]	Response as Incorporation of [^{3}H]TdR					
		ConA		PWM		WCI	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
1	Saline	74		33		4.30	
	Lysozyme	56	24	22	32	5.05	-17
	Neuraminidase	38	49	9	73	2.10	51
	CMRI + CMEI	15	80	3	91	0.93	78
2	Saline	38		15		1.34	
	NaOH	22	42	17	-13	2.12	-58
	HCl	22	42	7	53	1.38	-3
	Periodate	33	13	12	20	1.11	17
	CMRI + CMEI	12	68	3	80	2.51	62

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 80 µg of treated CMRI reconstituted with 20 µg of CMEI.

fractions used singly gave increased responses to one or more of the recall antigens (Table 2, Exp. 2).

Sensitivity of ISC to Enzymes, Acid, Base and Periodate

Enzymatic treatments were used to identify the chemical nature of the ISC. Treatments of WCI with DNase, RNase, proteinase K, thermolysin, beta-galactosidase, glucose oxidase, lipase, lysozyme, and neuraminidase were ineffective in the inactivation of the ISC (not shown). Treatments of CMRI with enzymes prior to reconstitution with CMEI demonstrated that the ISC could be partially inactivated with only lysozyme and neuraminidase (Table 3, Exp. 1). The most effective treatment was lysozyme. Neuraminidase treatment had a marginal effect on the ability of CMRI to reconstitute suppression with CMEI.

Treatment of the CMRI with periodate partially inactivated all suppression, whereas treatment of CMRI with NaOH and HCl inactivated PWM- and antigen-specific suppression, respectively (Table 3, Exp. 2). Treatment of WCI with these chemicals gave similar results (data not shown). Thus, the ISC may be separated into mitogen- and antigen-specific activities.

Sensitivity of ISC to Dithiothreitol

Since the ISC was not sensitive to protease treatment, the susceptibility of the active components to inactivation by a compound known to react with disulphide groups was tested. CMRI and WCI were treated with the reducing agent dithiothreitol and the reduced sulfhydryls blocked from reassociating by the addition of the alkylating agent, iodoacetamide (IA). Treated CMRI was reconstituted with CMEI prior to injection into mice.

Treatment of WCI with DTT and IA did not inactivate the ISC (Table 4, Exp. 1). However, reduction and alkylation of CMRI inactivated the ISC, resulting in spleen weights and lymphocyte responsiveness at or near control levels. Alkylation alone did not inactivate the ISC. Reduction unaccompanied by alkylation resulted in a marked increase in lymphocyte responsiveness to mitogen and antigen, but the activity did not reach the levels exhibited by saline-injected controls.

Reduction by DTT may cleave disulphide bonds inside the active complex and render it inactive. Alternatively, reduction may liberate the component of the active complex from the cell

Table 4

Effect of Dithiothreitol (DTT) and Iodoacetamide (IA) Treatment on the Ability of WCI and Reconstituted CMRI to cause Immunosuppression

Exp.	WCI/CMRI treatment[a]	Spleen Wt.[b]	Response as Incorporation of [^{3}H]TdR					
			ConA		PWM		WCI	
			SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
1	Saline	96.5 ± 10.1	94		37		1.49	
	DTT + IA	580.4 ± 30.3	11	88	1.5	96	0.62	58
	Untreated WCI	550.9 ± 35.7	9	90	1.5	96	0.55	63
2	Saline	83.5 ± 2.3	56		21		3.04	
	IA only	695.0 ± 66.2	8	86	2	90	0.58	81
	DTT only	209.4 ± 23.2	29	48	9	57	1.43	53
	DTT + IA	102.8 ± 6.1	50	11	20	5	3.39	-12
	Untreated CMRI	443.0 ± 73.6	11	80	2	90	0.97	68
	(CMRI + CMEI)[e] + DTT + IA	471.8 ± 94.3	12	79	2	90	1.01	67

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 80 µg of treated CMRI reconstituted with 20 µg of CMEI, or 100 µg of treated WCI.

matrix into the CMR supernatant fraction. To test these two possibilities, CMRI was reduced with DTT, and both the pellet and supernatant reconstituted with CMEI (Table 5). A factor able to reconstitute suppression with CMEI was present in the supernatant of DTT-treated CMRI. This factor was present in much smaller amounts in the CMRI supernatant of untreated samples. Interestingly, the activity of splenic lymphocytes from animals injected with normal CMRI supernatants was enhanced by recall antigen.

Reconstitution of CMRI with CMEs from Eukaryotes and Prokaryotes

We have observed that CMRI reconstituted with either CMEI or CMEII restored the biological activity of the ISC (Table 1, Table

TABLE 5

Comparison of the ISC Activity of C. burnetii Fractions after Dithiothreitol (DTT) Treatment of CMRI

Mice injected with[a]	DTT[f]	Response as Incorporation of [^{3}H]TdR					
		ConA		PWM		WCI	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
Saline		47		16		1.31	
CMRI pellet	+	6	87	1	94	0.76	42
CMRI super.	+	8	83	1	94	0.70	47
CMRI pellet	-	15	68	5	69	0.81	38
CMRI super.	-	31	34	13	19	2.82	-115

[a]Mice were given a single 0.5 ml i.p. injection of saline containing pelleted or supernatant material from 80 µg of treated CMRI reconstituted with 20 µg of CMEI.

2). To test the specificity of the C. burnetii CME, we studied the effect of CMEs from different sources. Thus, CMEs of L. pneumophilia, embryonated hen yolk sacs, and C57BL/10 ScN spleen cells were reconstituted with C. burnetii CMRI. All preparations tested, either bacterial or non-bacterial, were effective in reconstituting the ISC activity (Table 6).

Reconstituting of CMRI with Lipophilic Reagent-grade Lipids

Since different CMEs restored ISC activity, we designed additional studies to ascertain whether the active components contributed by CMEs were chemically unique. Several reagent-grade prokaryotic and eukaryotic membrane lipids were evaluated after reconstitution with CMRI.

Reconstitution of CMRI with phosphatidyl serine, phosphatidyl choline, phosphatidic acid, cholesterol, cardiolipin, phosphatidyl

TABLE 6

Comparison of Different CMEs to Reconstitute the Activity of the ISC

Exp.	Mice injected with[a]	Spleen Wt.[b]	Response as Incorporation of [^{3}H]TdR ConA SI[c]	ConA % Supp.[d]	PWM SI	PWM % Supp.	WCI SI	WCI % Supp.
1	Saline	119.6 ± 7.6	45		22		1.70	
	CMRI + Leg. CME	306.4 ± 32.0	11	76	2	91	0.43	75
2	Saline	113.7 ± 4.7	29		13		1.46	
	CMRI + YS-CME	433.7 ± 63.5	10	66	4	69	0.41	72
3	Saline	91.2 ± 9.2	46		21		3.73	
	CMRI + spleen CME	253.8 ± 72.0	13	72	2	90	0.74	80

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 80 µg of CMRI reconstituted with 20 µg of the respective CME.

glycerol, phosphatidyl glycerol dioleoyl, phosphatidyl ethanolamine, diphosphatidyl ethanolamine dipalmitoyl, *E. coli* KDO, and C-15 and C-17 fatty acids restored the activity of the ISC (Table 7). Glycerol and glycerol phosphate did not restore the ISC activity when reconstituted with CMRI, as there was a significant increase in activity in the presence of recall antigen.

Adsorption of Active Components of CME

The amphipathic nature of the components contributed by CMEI was demonstrated by passing CMEI in a chloroform-methanol solvent through solid-phase extraction columns. When the eluates were reconstituted with CMRI, the diol and octyl columns most

TABLE 7

Comparison of Reagent-grade Lipids to Reconstitute the Activity of the ISC

Exp.	Mice injected with[a]	Spleen Wt.[b]	Response as Incorporation of [^{3}H]TdR					
			ConA		PWM		WCI	
			SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
1	Saline	92.7 ± 1.8	23		13		2.15	
	CMRI + phosphatidyl serine	337.6 ± 103.1	2	91	0.4	97	0.11	94
	CMRI + phosphatidyl choline	287.2 ± 64.2	5	78	2	85	0.60	70
2	Saline	110.6 ± 3.1	102		27		2.10	
	CMRI + phosphatidic acid	523.7 ± 59.1	6	94	0.7	97	0.35	83
	CMRI + glycerol phosphate	124.0 ± 23.8	85	17	29	-7	4.03	-92
	CMRI + glycerol	162.1 ± 58.3	75	26	24	11	3.02	-44
	CMRI + cholesterol	525.9 ± 64.5	7	93	1	96	0.53	75
	CMRI + Cardiolipin	559.8 ± 31.8	5	95	1	96	0.28	87
	CMRI + KDO	187.3 ± 37.2	37	64	7	74	1.20	43
	CMRI + C-15 fatty acid	137.6 ± 7.2	27	74	13	52	1.54	27
	CMRI + C-17 fatty acid	159.1 ± 4.7	62	39	11	59	1.77	16
3	Saline	105.7 ± 5.9	95		50		8.59	
	CMRI + phosphatidyl glycerol	291.7 ± 5.3	33	65	7	86	1.82	79
	CMRI + phosphatidyl glycerol dioleoyl	326.3 ± 8.4	14	85	2	96	1.01	88
	CMRI + phosphatidyl ethanolamine	373.6 ± 6.4	19	80	4	92	0.92	89
	CMRI + phosphatidyl ethanolamine dipalmitoyl	212.4 ± 7.0	22	77	6	88	1.34	84

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 20 µg of reagent grade lipid reconstituted with 80 µg of CMRI.

TABLE 8

The Effect of Passing CMEI Through Silica Gel Solid Phase Extraction Columns Prior to Reconstruction with CMRI and Injection into C57BL/10 ScN Mice.

CMEI treatment[a]	Spleen Wt.[b]	Response as Incorporation of [^{3}H]TdR					
		ConA		PWM		WCI	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
Saline	107.1 ± 7.4		17		1.81		
Diol	132.9 ± 8.6*[g]	33	-32*	14	18*	1.75	3*
C-18	293.7 ± 36.1	12	52	2	88	0.36	80
$1^o,2^o$ NH_2	262.8 ± 34.5	13	48	3	82	0.35	81
NH_2	267.3 ± 60.0	14	44	4	76	0.71	61
Sulfonic acid	407.8 ± 14.7	6	76	1	94	0.49	73
Silica	293.2 ± 3.1	7	72	2	88	0.27	85
N^+	358.8 ± 19.2	8	78	2	88	0.38	79
Phenyl	175.6 ± 55.0	16	36	6	65*	0.71	61
CN	210.9 ± 16.1	10	60	4	76	0.60	67
C-8	123.3 ± 34.3*	29	-16*	10	41*	0.44	76
CMEI	225.6 ± 4.2	14	44	3	82	0.60	67

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 80 µg of CMRI reconstituted with 20 µg of eluates or CMEI.

effectively adsorbed the active components of the ISC (Table 8). When injected into mice, the reconstituted eluate suppressed lymphocyte responses to a much smaller degree than reconstituted untreated CMEI and eluates of other adsorbents. Interestingly, the diol-adsorbed components were active in both mitogen and antigen suppression, whereas the C-8-adsorbed components were active in only T-cell suppression.

ISC of Different Strains of Coxiella

Eight strains of different virulence were evaluated for their ability to cause pathogenic reactions after injection of either viable or killed cells into C57BL/10 ScN mice (Table 9 and 10). Viable or killed CBHENI, CB9MIC7, and CB9MI514 induced pathological reactions. The injection of killed cardiac valve isolates, CBPAVI and CBKAVI, induced no increase in spleen weights, no significant T-cell suppression, significant enhancement of PWM responsiveness, and a decrease in antigenic negative modulation (Table 10). However, infection by CBPAVI and CBKAVI indicated that only PAVI induced significant pathological reactions. The pathological reactions induced by the ISC were not detected in animals injected with viable or killed phase II preparations. Infection of mice by AUSTII produced suppression of Con A and PWM responsiveness without the induction of antigenic negative modulation. Whereas infection of mice by M44II and 9MIIC4 increased lymphocyte response to Con A and antigen, the injection of killed antigens (AUSTII, M44II, or 9MIIC4) produced antigen-specific, negative modulation with AUSTII and M44II. We observed either no change or significant enhancement for the Con A and PWM responses.

A preparation of CMR and CME from the CBKAVI strain was tested for the ability to reconstitute the ISC. When CMR (CBKAVI) was reconstituted with CME from the 9MIC7 strain, the activity of the ISC was not detected (data not shown). But the CME (CBKAVI) was effective in restoring the ISC activity to the CMR from the 9MIC7 strain (data not shown). These results suggest that the

TABLE 9

Immunomodulation by Eight Different Strains of Viable C. burnetii After Infection of C57BL/10 ScN Mice

Mice injected with[a]	Spleen wt (mg)[b]	Response as Incorporation of [3H]TdR					
		ConA		PWM		WCI	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
Saline	85	21		12		2.9	
HENI	875*[h]	1	95	2	83	0.2	93
9MIC7	912*	1	95	1	92	0.5	83
9MI514	853*	3	86	5	58	0.7	76
PAVI	248	13	38	4	67	1.4	52
KAVI	167	23	-10	8	33	3.6	-24
AUSTII	121	15	29	5	58	2.9	0
M44II	159	25	-19	11	8	4.3	-48
9MIIC4	157	28	-33	11	8	4.7	-62

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 10^5 viable C. burnetii

TABLE 10

Immunomodulation by Eight Different Strains of Killed C. burnetii After Injection of C57BL/10 ScN Mice

Mice injected with[a]	Spleen wt (mg)[b]	Response as Incorporation of [3H]TdR					
		ConA		PWM		WCI	
		SI[c]	% Supp.[d]	SI	% Supp.	SI	% Supp.
Saline	92	15		5		3.1	
HENI	228*[h]	12	20	4	20	0.6	81
9MIC7	462*	5	67	1	80	0.2	96
9MI514	739*	8	47	1	80	0.2	96
PAVI	91	16	-7	11	-120	2.0	35
KAVI	105	13	13	9	-80	2.2	29
AUSTII	106	13	13	8	-60	1.7	45
M44II	101	20	-33	7	-40	2.1	32
9MIIC4	80	22	-47	13	-160	2.6	14

[a]Mice were given a single 0.5 ml i.p. injection of saline containing 100 µg of killed C. burnetii.

CBKAVI strain was defective in the synthesis of an ISC component contributed by CMRI or of a cell matrix receptor which anchors a component of the CME to produce an active ISC.

DISCUSSION

Coxiella burnetii is able to modulate the host immune response in positive and negative directions. These bivalent responses of the host may be both specific and non-specific. While animals injected with phase I cells show increased tumoricidal and bactericidal abilities (2), pathogenic reactions such as hepatomegaly, splenomegaly, liver necrosis, death, lymphocyte hyporesponsiveness, and antigen-specific negative modulation also occur (4,5,6). These adverse responses generated by the components of the ISC were the subject of the current study.

Inactivated phase I C. burnetii cells are effective in inducing immunosuppression in mice. Therefore, this activity is not the result of an infectious process, but of some microbial component. Phase I cells at low concentrations do not exert a toxic effect on lymphocytes cultured in vitro (5,6) but, at high concentrations (>100µg/ml), lymphocytes are killed. In fact, 5 µg/ml of WCI as in vitro recall antigen stimulated lymphocytes from saline-injected mice. However, when used as recall antigens with cultured lymphocytes from WCI or reconstituted CMRI-injected animals, substantial immunosuppression occurred. The CMRI is non-toxic at high concentrations (>100µg/ml) and is mitogenic (6). Therefore, structural features of the ISC were responsible for the

induction of suppression in vivo and the initiation of lymphocyte mitogenesis in vitro.

The ability of C. burnetii to induce immunosuppression appears to be a property of phase I cells and of the reconstituted CMRI. A major difference between phase I and phase II organisms is reflected by their LPS structure. Phase I LPS is "smooth" while phase II LPS is of the "rough" chemotype (7,15). However, phase I LPS is not the sole contributor to pathophysiological changes and immunosuppression in this murine system (6). Neither proteinase K-treated LPSI injected alone or reconstituted with CMEI induced pathological reactions to the same degree as reconstituted CMRI, which contains LPSI. It is possible, however, that LPSI in association with other biologically active molecules contributed in some fashion to the activity of the ISC.

The antigen-specific nature of the immunomodulation suggests that specific structural determinants of the ISC are required for the initiation of the pathogenic reactions. We observed phenomenon of antigen-specific suppression after lymphocytes primed in vivo by WCI or reconstituted CMRI were stimulated in vitro by specific recall antigens. These results suggest that lymphocyte hyporesponsiveness is not simply a "failure to respond" to mediators in culture, but an event requiring prior exposure to antigen. The observed immunosuppression after the injection of WCI may be due, in part, to an imbalance in different spleen cell populations between experimental and control mice. The negative modulation of primed lymphocytes by antigen in culture is a

hallmark of this suppression mechanism. The kinetics of the in vivo development of suppressors and imbalances in cell types is currently under study.

The architectural association of the components of the ISC appears to be localized with the C. burnetii cell wall. Analysis of the chemical nature of the components of the ISC by conventional means was not possible. Therefore, techniques such as high pressure liquid chromatography and other chromatographic procedures could not be performed on the insoluble matrix. We subjected the WCI and the CMRI to procedures designed to inactivate or extract the components of the ISC in situ. When WCI was treated with enzymes having protein, carbohydrate, and lipid specificities, no treatment resulted in a statistically significant decrease in the activity of the ISC.

Exposure of critical structural determinants of the ISC was accomplished by CM extraction. Phase I CMR fractions of C. burnetii were susceptible to lysozyme and neuraminidase. Proteinase K and thermolysin were unsuccessful in reducing the level of immunosuppression in treated CMRI samples, suggesting that susceptible proteins did not participate in the activity of the ISC. The effectiveness of lysozyme treatment of CMRI in diminishing the activity of the ISC implies a possible involvement of peptidoglycan. The susceptibility of the CMRI suppressive component to neuraminidase treatment suggests that sialic acid may play a role in the activity of the ISC.

Although the active components of the CMRI ISC exhibited sensitivity to certain enzymes which hydrolyze carbohydrates,

there remained a possibility that proteinase-resistant proteins were also structurally involved. In fact, proteinase-resistant proteins have been identified in Coxiella (16,17). When CMRI was treated with DTT and IA prior to reconstitution with CMEI and subsequently injected into mice, lymphocyte responsiveness was not significantly different from that of control, saline-injected animals. Thus, disulphide-bonded components may be an intrinsic or extrinsic component of the CMRI ISC. Lipophilic components of CMEI effectively shielded the ISC from reduction and alkylation because neither WCI nor reconstituted CMRI was susceptible to these treatments. Therefore, both chemical and architectural associations are important in the complex interactions of the components of the ISC.

The elimination of suppression after treatment of CMRI with DTT provides clues regarding the location in the cell wall and the association of these components in the ISC. Recovery of an active component in the supernatant of DTT-treated CMRI suggests that disulphide groups are actively involved in anchoring the DTT-soluble component to the cell matrix. In the biologically active complex, the disulphide linkage would likely attach the components of the ISC to the cell wall. Furthermore, the fact that alkylation of the DTT-extracted CMR prevented reassociation of the ISC suggests that sulphydryl groups may be extrinsic to the active center but intrinsic to the fully active ISC.

The lipophilic properties of the CMEs suggested that perhaps any lipophilic molecule could provide the appropriate

configuration for the expression of the ISC activity. Therefore, sources of CMEs other than C. burnetii were evaluated for their ability to reconstitute the activity of the ISC with CMRI. The CMEs from L. pneumophilia, hen yolk sacs, and C57BL/10 ScN spleen cells were each able to reconstitute the activity of the ISC. That such a diverse assortment of cell types containing many chemical entities reconstituted the activity of the ISC with CMRI implies that the CME component(s) may be rather ubiquitous molecules(s). These lipophilic molecules may have a common physical characteristic, such as amphipathy, that is required for molecular associations. Several reagent-grade membrane lipids were as effective as CMEs in the reconstitution of the ISC activity. While the suppressive component contributed by C. burnetii CMEI may be unique in structure, the major determinant(s) of successful reconstitution is likely the hydrophobic components of the different CMEs.

We studied the amphipathic and electrostatic nature of the CMEI suppressive component by passing CMEI through columns containing silica complexed to various adsorbents. Diol and octyl were the only adsorbents consistently able to adsorb the suppressive component(s) contributed by CMEI. In normal phase liquid chromatography, the diol group adsorbs hydrophilic compounds with cis-hydroxyl groups, whereas association with octyl is hydrophobic. Components of CMEI retained by the diol column were effective in the induction of T-cell and antigen-specific

suppression, while components eluted from the octyl column were effective in only antigen-specific suppression. Therefore, the CMEI suppressive component(s) may be amphipathic in nature. Alternatively, several molecules, each hydrophilic or hydrophobic, may contribute to the CME suppressive component(s). This possibility is least likely due to the general non-polar nature of CMEs.

The expression of the ISC activity by different isolates of C. burnetii was tested. A survey of eight C. burnetii strains with structural variation in LPS (7) indicated that phase I Henzerling, phase I Nine Mile, and phase I Nine Mile 514 expressed the ISC activity. The phase I Ohio strain was previously shown to express the ISC activity (4,5,6). Although the phase I Nine Mile 514 strain carries the ISC, it is a semi-rough LPS chemotype and does not express any rough LPS (7). Interestingly, the phase I cardiac valve isolates, "KAV" and "PAV" strains, which express both the smooth (phase I), semi-rough and rough (phase II) LPS (7), expressed significantly different properties of the ISC activity. This is a further indication that the LPS may not be a major determinant of the ISC. Although organisms expressing the smooth and semi-rough LPS induce the ISC activity, not all organisms classified as phase I induce suppression in the murine system. Noteworthy was the diminution of ISC activity in strains possessing marked increases in the concentration of the rough LPS chemotype. Although some of these strains expressed both the smooth and semi-rough LPS, they all expressed significant amounts

of the rough LPS chemotype. Thus, the synthesis of LPS and expression of the ISC appear to be coordinately controlled and may reflect linkage on the chromosome, possibly under genetic control. The correlation between LPS structural variation (7) and ISC activity may be incidentally related to the cell matrix receptor involved in anchoring the DTT-soluble and CME active components.

The immunomodulation described in this study with a murine model for C. burnetii-induced pathogenesis has allowed us to dissect partially the architectural and biochemical properties of the ISC. The ISC activity shows significant variation among C. burnetii strains. Strains expressing only smooth LPS carried the most potent ISC activity. None of the strains with only rough LPS expressed the ISC activity. Also the CMRs of phase II strains with rough LPS were ineffective when combined with CMEs from smooth strains. In addition, the CMR from the KAVI cardiac valve isolate was ineffective when combined with CMEs from strains expressing the ISC activity. Although the cardiac valve isolates apparently induce antigen-specific suppression in patients (18), these strains were not effective in the murine model. Inferentially, the determinants which induce antigen-specific suppression in patients may be different from those of the ISC assayed in the murine model. Alternatively, remnants of the ISC may be as effective in humans as the complete complex is in mice. The differences observed between human and mice may reflect kinetic differences as human patients have harbored the organism

for several years, and mice were exposed for only 14 days in these studies. Furthermore, these differences may reflect dissimilarities in the host response to the antigen-specific properties of the ISC.

A putative schematic model can be proposed for the active components of the phase I ISC (Fig. 1). This model consists of at least three fractions which contain active components: 1) components extracted with CM (4:1); 2) components extracted with DTT; and 3) components remaining attached to the cell matrix. Component 1 is not covalently linked to any of the remaining components and may shield the complex in situ from the effects of chemical and enzymatic degradation. Components 2 and 3 form an integral part of CMRI and may be linked by disulphide bonds. Component 3 may be a cell matrix receptor which possibly functions as an anchor for 2. A reconstituted mixture of components 1 and 2 is required for the biological activity of the ISC. These components may associate via hydrophobic interactions. Component 2 is specific to phase I C. burnetii whereas component 1 may be ubiquitous. The putative model of the C. burnetii CMRI suppressive components may bear compositional similarities to a Mycobacterium leprae glycolipid, which is capable of inducing suppression of mitogenic responses of lepromatous patients' lymphocytes in vitro (19). Suppressor cells have been shown to have a rather exquisite specificity for the terminal trisaccharide of the phenolic glycolipid of M. leprae (20). Suppressor cell-inducing epitopes have also been demonstrated in the lysozyme

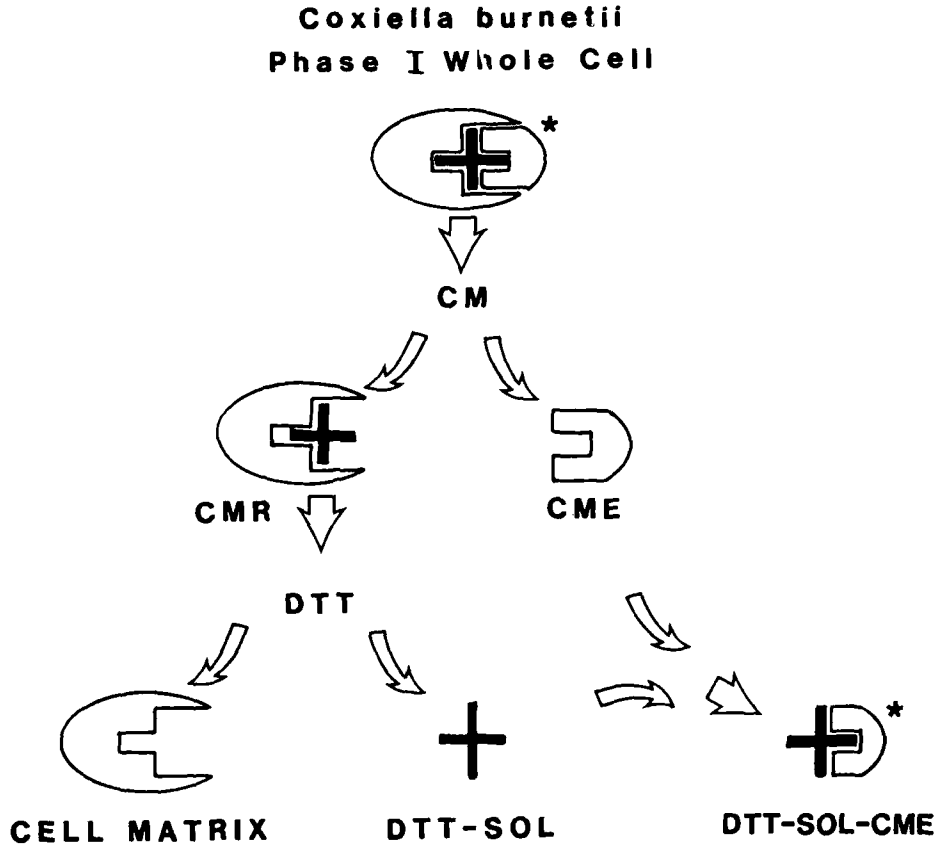

Fig. 1. Putative model of the Coxiella burnetii ISC. The components of this model may be segregated into three parts: 1. non-covalent, lipophilic components in the CME; 2. DTT-soluble, covalently associated components which attach to the cell matrix; 3. cell matrix receptor for 2 which anchors 1 plus 2 to the cell surface. *Represent active fractions of the ISC. The components singly do not appear to be active in the murine model.

system (21). Further purification and chemical analyses of the components of the ISC are required before we can understand the complex interaction between C. burnetii and the host.

ABBREVIATIONS

CBAUSTII	-	C. burnetii phase II Australian strain
CBHENI	-	C. burnetii phase I Henzerling strain
CBKAVI	-	C. burnetii phase I K (aortic valve) strain
CBM44II	-	C. burnetii phase II M44 strain
CB9MIC7	-	C. burnetii phase I Nine Mile clone 7 strain
CB9MI514	-	C. burnetii phase I Nine Mile 514 strain
CB9MIIC4	-	C. burnetii phase II Nine Mile clone 4 strain
CBOI	-	C. burnetii phase I Ohio strain
CBPAVI	-	C. burnetii phase I P (aortic valve) strain
CME (I,II)	-	Chloroform methanol extract (phase I, phase II)
CMR (I,II)	-	Chloroform methanol residue (phase I, phase II)
Con A	-	Concanavalin A
ISC	-	Immunosuppressive complex
KDO	-	2-Keto-3-deoxyoctonic acid
LPS	-	Lipopolysaccharide
PBS	-	Phosphate-buffered saline
WC (I,II)	-	Whole cell (phase I, phase II)

LEGEND

[b]Mean spleen weight in milligrams ± one standard deviation. All underlined values are statistically different from control values at the 95% confidence level.

[c]Stimulation index = $\frac{\text{CPM in mitogen or antigen-stimulated cultures}}{\text{CPM in unstimulated cultures}}$

[d]% suppression = $(1 - \frac{\text{SI of cells from mice injected with antigen}}{\text{SI of cells from saline-injected controls}}) \times 100$.

[e]Reconstitution prior to treatment.

[f]CMRI was incubated at room temperature for 2 hr in the presence (+) or absence (-) of 0.1 M DTT in saline and pellets and supernatants collected.

[g]All values indicated with (*)are significantly less than suppresssive levels of reconstituted untreated CMEI at the 95% confidence level.

[h]*indicates that livers contained extensive surface necrotic lesions.

Note: All underlined values are statistically different from control values at the 95% confidence level.

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